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Longitudinal variability of diazotroph abundances in the subtropical North Atlantic Ocean

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Diazotrophy-related studies in the North Atlantic have largely focused on its western tropical area, leaving the subtropics and the east undersampled. We studied the longitudinal distribution of *Trichodesmium*, UCYN-A, UCYN-B, the putative Gammaproteobacterium γ-24774A11 and *Richelia* (Het1) along 24.5°N, using quantitative polymerase chain reaction on different size fractions (10, 10–3 and 3–0.2 μm) and additional filament counts for *Trichodesmium*. *Trichodesmium* was the most abundant phylotype, followed by UCYN-A, γ-24774A11 and Het1, with maximum abundances of 8.8×10^5, 2.0×10^5, 3.3×10^3 and 3.4×10^2 *nifH* copies L^{-1}, respectively, whereas UCYN-B was mostly undetected. A clear shift in the diazotroph community was observed at ~30°W, coinciding with the transition between the North Atlantic Subtropical Gyre boundary and inner core. This transition zone divided the transect into an eastern half dominated by UCYN-A and western half dominated by *Trichodesmium* and γ-24774A11. γ-24774A11 was only detected in the 10–3 μm fraction, suggesting their association with larger microbes or aggregates. Our results indicate that typical size fractionation by 10 μm is not optimal for reconciling diazotroph phylotypes to N_2 fixation rates and that non-cyanobacterial diazotrophs may contribute importantly to bulk diazotrophic activity in the western subtropical North Atlantic.

KEYWORDS: *nifH*; qPCR; N_2 fixation; upwelling; North Atlantic Subtropical Gyre

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INTRODUCTION

The biological fixation of atmospheric dinitrogen (N_2) provides significant inputs of reactive nitrogen to aquatic ecosystems and hence contributes to phytoplankton primary production (Karl *et al.*, 2002). This process is performed by specialized prokaryotes called diazotrophs, distributed among broad phylogenetic groups from cyanobacteria and other bacteria to archaea (Zehr *et al.*, 2003). The occurrence of N_2 fixation in the ocean was first described in the North Atlantic basin and attributed to the filamentous cyanobacterium *Trichodesmium* (Dugdale *et al.*, 1961). In the following decades, it has become apparent that the diazotrophic community is also composed of diatom-diazotroph symbioses (DDAs), unicellular cyanobacteria (UCYN, divided into three groups UCYN-A, -B, -C) and non-cyanobacterial diazotrophs (including both bacteria and archaea) (Zehr *et al.*, 2003). N_2 fixed by UCYN and DDAs at times locally exceeds that of *Trichodesmium*, highlighting the importance of these diazotrophs in N_2 fixation studies (e.g. Ratten *et al.*, 2015).

The different diazotrophic phylotypes are heterogeneously distributed in the North Atlantic Ocean because of geographical differences in the environmental factors that control them (Benavides and Voss, 2015). *Trichodesmium* is known for needing nitrate-poor, warm, stratified waters and is abundant in the tropical western Atlantic where these conditions persist (Capone *et al.*, 2005). UCYN-A are small photoheterotrophic cyanobacteria (<1 μm) not capable of carrying out the oxygen-evolving steps of photosynthesis or fixing inorganic carbon (Zehr *et al.*, 2008), and they are commonly found in symbiosis with a eukaryotic alga (Thompson *et al.*, 2012). UCYN-A seem to be able to fix N_2 at relatively low temperatures and high inorganic nitrogen concentrations (e.g. <22°C and >1.5 μM) (Turk *et al.*, 2011; Ratten *et al.*, 2015). UCYN-B are restricted to warm tropical waters (e.g. Moisander *et al.*, 2010; Turk *et al.*, 2011), whereas UCYN-C are often associated with coastal areas (Langlois *et al.*, 2008; Turk *et al.*, 2011). DDAs need a source of silica for the synthesis of the frustule of the diatom host; thus silica in the river plumes of the Amazon and Congo rivers may promote them (Foster *et al.*, 2007; 2009; Subramaniam *et al.*, 2008). The distribution of non-cyanobacterial diazotrophs in the North Atlantic seems to be extensive, spanning a wide latitudinal range (Luo *et al.*, 2012), although their ecology and environmental constraints are unknown (Farnelid *et al.*, 2011), partly because of the difficulties in discerning cyanobacterial from non-cyanobacterial N_2 fixation rates in $^{15}N_2$ tracer incubations (Benavides and Voss, 2015). Diazotrophic bacteria from the open ocean cluster with a wide range of phylogenetic groups, including Firmicutes, Alpha-, Beta-, Gamma- and Deltaproteobacteria (Zehr *et al.*, 2003). The Gammaproteobacteria-affiliated phylotype γ-24774A11 was recovered from the South China Sea (Moisander *et al.*, 2008), and the same diazotroph phylotype (also called Gamma A or UMB) has been recovered at high frequencies from several environments such as the Atlantic, Pacific and Indian Oceans and the Mediterranean Sea (Bird *et al.*, 2005; Church *et al.*, 2005a; Turk *et al.*, 2011; Moisander *et al.*, 2014; Shiozaki *et al.*, 2014; Langlois *et al.*, 2015). At least three different published quantitative polymerase chain reaction (qPCR) primer sets target this phylotype cluster that has some microdiversity (Moisander *et al.*, 2014), and these primers have been termed Gammaproteobacteria (Church *et al.*, 2005a), Gamma-A (Langlois *et al.*, 2008) and γ-24774A11 (Moisander *et al.*, 2008). Each of these three primer sets targets a slightly different variant in the cluster (Moisander *et al.*, 2014).

The distribution and abundance of all of these diazotrophic phylotypes have been previously reported in the North Atlantic by qPCR assays of the *nifH* gene (which encodes for the nitrogenase iron protein component of the nitrogenase enzyme complex) (e.g. Langlois *et al.*, 2008; Foster *et al.*, 2009; Goebel *et al.*, 2010). There is a geographic bias in the distribution of these studies, with more samples taken in the western than in the eastern North Atlantic Ocean (Montoya *et al.*, 2007). Moreover, more sampling has been conducted in the tropics compared with higher latitudes, leaving the subtropical and temperate latitudinal zones with scarce data (Benavides and Voss, 2015). Most *nifH* data available in the North Atlantic are restricted to the latitudinal band located between the Equator and ~15°N, and there is a wide gap in observations in the areas comprised by 20–40°N and 40–80°W, as can be observed in Fig. 3 of Luo *et al.* (2012). This gap impedes a comprehensive assessment of the spatial distribution of different diazotroph species in the North Atlantic.

With the aim of contributing to the few published studies on the longitudinal variability of diazotrophy in the North Atlantic (Voss *et al.*, 2004; Montoya *et al.*, 2007), we quantified the abundance of the main diazotrophic phylotypes along 24.5°N from 10 to 80°W and examined their geographical distributions in parallel with different environmental conditions and phylotype-specific constraints.

METHOD

Hydrography, nutrients and chlorophyll *a*

The leg 8 of the Malaspina circumnavigation cruise took place from 27 January to 15 March 2011 onboard the

R/V *Sarmiento de Gamboa*, sailing westwards from the Northwest African coast to the North American coast along 24.5°N (Fig. 1). Temperature, salinity and chlorophyll-fluorescence data were recorded with a SeaBird 911 plus CTD equipped with a Sea-Tech fluorometer. The CTD was mounted on a General Oceanics rosette frame equipped with 24 Niskin bottles (12 L volume).

Samples for nutrient analysis were taken with the 12 L Niskin bottles, stored in 15 mL polypropylene tubes and immediately frozen at −20°C until analysis ashore. The concentrations of nitrate plus nitrite, phosphate and silicate were determined with a Technicon segmented-flow autoanalyser. Standard methods were modified to obtain a detection limit of 2 nmol L^{-1} (Raimbault *et al.*, 1990). Details on the concentrations and distributions of nutrients can be found elsewhere (Benavides *et al.*, 2013b; Mompeán *et al.*, 2013) and will be used here only with the purpose of interpreting the distribution of diazotrophs.

The mixed layer depth (MLD) was estimated from an increase in water column density (σ_t) of 0.125 kg m^{-3} with respect to surface values (Benavides *et al.*, 2013a). Chlorophyll *a* (Chl *a*; mg m^{-3}) data were obtained from the National Aeronautics and Space Administration (NASA) Goddard Earth Sciences Data and Information Services Center Giovanni (NASA GES DISC) online database and averaged for the months of February and March 2011.

DNA collection, extraction and qPCR assays

Samples for DNA were taken at 15 stations along the transect (Table I). Surface seawater samples (~5 m) were collected using a 30 L Niskin bottle. Ten litres were transferred to acid-washed darkened carboys with a barbed faucet. Acid-washed vacuum tubing was connected to the faucet, and three separate filter holders (Whatman) were connected in-line containing 47 mm diameter white 10, 3 and 0.2 μm polycarbonate filters (GE-Osmonics Poretics). The end of the line was connected to a water jet aspirator vacuum pump (Eyela). The fractions retained were thus >10, <10−>3 (hereafter abbreviated as 10−3 μm) and <3−>0.2 μm (hereafter abbreviated as 3−0.2 μm), respectively. After filtration, the filters were transferred to sterile screwcap cryovials and stored at −80°C until analysis.

DNA was extracted using the DNeasy Plant Mini Kit (Qiagen), as modified by Moisander *et al.* (2008). The abundance of diazotrophs was determined using TaqMan qPCR assays and previously published primer−probe sets for *Trichodesmium*, UCYN-A (Church *et al.*, 2005a), UCYN-B (Moisander *et al.*, 2010), the Gammaproteobacterium γ-24774A11 (Moisander *et al.*, 2008) and Het1 (*Richelia-Rhizosolenia* DDAs) (Church *et al.*, 2005b). *Trichodesmium* trichomes usually measure >80 μm and were therefore only analysed on the >10 μm fraction. Het1 on the >10 and 10−3 μm fractions, UCYN-A and UCYN-B were analysed in the 10−3 and 3−0.2 μm fractions, and the γ-24774A11 were analysed in all three size fractions.

The qPCR was run in 20 μL reactions that consisted of 10 μL ABI TaqMan Gene Expression Master Mix, 6.4 μL nuclease-free water, 0.5 and 0.25 μM final concentrations of primers and probe, respectively, and 1.6 μL DNA template. All samples were run in duplicate. Ten-fold dilutions of linearized plasmids containing the relevant *nifH* targets were used as standards and were included with each 96-well plate run. The reactions were run on a StepOnePlus Real-Time PCR system (Life Technologies). Standard curves were made by plotting linear regression of the threshold cycle (C_t) and log gene copies per reaction using duplicate standards ranging from 10^8 to 10^0 gene copies. Amplification efficiencies were >90% for all reactions. Duplicate no template control wells were included in all plates run and did not show amplification in any runs. Inhibition tests were carried out for all samples by combining the sample and 1.6 μL of 10^5 standard as a template. The efficiencies of inhibition tests ranged from 97.3 to 101.73%, and thus we consider that our samples were not inhibited. The limit of detection (LOD) and detected but not quantifiable (DNQ) limits used were one and eight gene copies per reaction, respectively. Samples that were below LOD were designated a value of 0 in the data set, whereas gene copies higher than LOD but less than DNQ were designated a conservative value of 1 *nifH* gene copy per litre.

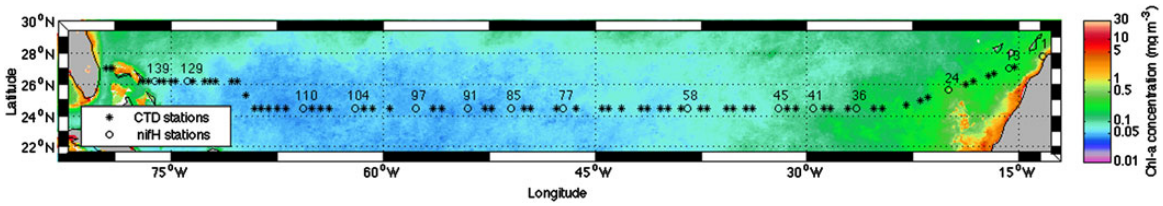

Fig. 1. Map of stations where CTD profiles were performed ("CTD stations", labelled with an asterisk) and stations where samples for *nifH* analyses were taken ("*nifH* stations", labelled with an open symbol and station number), superimposed on chlorophyll *a* concentration data (Chl a; mg m^{-3}).

Table I: Measured values of core parameters (temperature, salinity and fluorescence) and inorganic nutrient concentrations (nitrate plus nitrite - $NO_3^-+NO_2^-$ - , silicate - SiO_2^- , and phosphate - PO_4^{3-} - ; Benavides et al., 2013b; Mompeán et al., 2013) at stations where diazotroph phylotypes were surveyed

Station	Date (dd/mm in 2011)	Latitude (°N)	Longitude (°W)	Surface temperature (°C)	Surface salinity	Surface fluorescence (relative units)	MLD (m)	$NO_3^-+NO_2^-$ (μM)	PO_4^{3-} (μM)	SiO_2 (μM)	>10 μm N_2 fixation rates (nmol N L^{-1} h^{-1})[a]	<10 μm N_2 fixation rates (nmol N L^{-1} h^{-1})[a]
1	28/01	27.78	13.34	19.59	36.77	1.61	75.50	12.47	0.73	19.34	0.385	0.335
13	29/01	27.02	15.70	20.70	36.96	1.20	87.41	0.05	0.58	0.66	0.210	0.167
24	01/02	25.63	20.00	21.73	37.06	1.19	123.16	0.07	0.65	0.58	0.191	0.143
36	05/02	24.50	26.54	21.57	37.24	1.05	129.12	0.11	0.20	0.12	0.261	0.237
41	07/02	24.50	29.59	22.44	37.42	0.93	123.16	0.01	0.58	0.86	0.302	0.181
45	09/02	24.50	32.04	22.81	37.45	0.92	99.33	0.01	0.65	0.79	0.271	0.151
58	13/02	24.50	38.49	23.07	37.53	0.85	117.21	0.13	0.39	0.74	0.178	0.018
77	18/02	24.50	47.27	23.41	37.39	0.86	93.37	0.05	0.42	0.20	0.243	0.116
85	21/02	24.50	50.96	23.99	37.24	0.76	81.46	n/a	n/a	n/a	0.093	0.023
91	23/02	24.50	54.03	24.26	36.99	0.79	57.62	n/a	n/a	n/a	0.104	0.041
97	25/02	24.50	57.72	23.99	36.73	0.80	39.74	n/a	n/a	n/a	0.108	0.051
104	27/02	24.50	62.01	24.92	36.28	0.83	63.58	n/a	n/a	n/a	0.142	0.065
110	01/03	24.50	65.68	23.82	36.73	0.76	57.62	n/a	n/a	n/a	0.168	0.088
129	07/03	26.20	73.82	23.81	36.78	1.07	51.66	0.19	0.16	0.12	0.024	0.033
139	09/03	26.20	76.15	22.69	36.77	0.89	75.50	0.12	0.17	n/a	0.089	0.061

n/a indicates data not available.
[a]From Benavides *et al.* (2013b).

Trichodesmium filament counts

Samples were collected by towing a 40 μm mesh size plankton net from 200 m to the surface at 43 stations (Mompeán *et al.*, 2013), from which 6 stations (24, 45, 85, 91, 97 and 104; Fig. 1) were coincident with those where DNA was sampled. The volume of seawater filtered at each station was 14 m^3. *Trichodesmium* filament counts were made on aliquots of the sample preserved in glutaraldehyde (25% final concentration) using a FlowCam system (Fluid Imaging Technologies), following the recommendations by Álvarez *et al.* (2014). Samples were run in the autoimage mode using a 4× lens (40× overall magnification) and a flow cell of 300 μm depth. Prior to analysis, the samples were screened with a 100 mm nylon mesh to prevent clogging of the FlowCam cell. Results are reported as number of trichomes per litre of seawater.

N_2 fixation rates

The fractionated (>10 and <10 μm) N_2 fixation was assayed with the dissolved $^{15}N_2$ method according to Mohr *et al.* (2010), as described in Benavides *et al.* (2013b).

RESULTS

Hydrography, nutrients and Chl *a* data

Temperature and salinity were low near the coastal upwelling off Northwest Africa (~19°C and 36.8, respectively; Table I). From east to west, temperature increased from ca. 20 to ca. 25°C at 62°W and then decreased slightly (to ~23°C) until the westernmost station. Fluorescence values were highest off the Northwest African coast (Table I) and decreased westwards mirroring the distribution of Chl *a* (Fig. 1). The surface concentrations of nitrate plus nitrite, phosphate and silicate were maximal off the Northwest African coast (12.61, 0.73 and 19.34 μM, respectively; Table I) and also decreased westwards along the transect. Further details on hydrographic variables measured along the transect can be found elsewhere (Benavides *et al.*, 2013b; Mompeán *et al.*, 2013). The MLD was ~80 m in the eastern end of the transect, and it deepened in the central part of the North Atlantic Subtropical Gyre (NASG), shoaling towards the western end of the transect with values ~60 m (Table I). N_2 fixation rates (from Benavides *et al.*, 2013b) were in a similar range in the >10 μm fraction (0.009–0.385 nmol N L^{-1} h^{-1}) and in the <10 μm fraction (0.011–0.335 nmol N L^{-1} h^{-1}), although the former were generally higher than the latter along the transect. N_2 fixation rates were highest off the Northwest African coast and maintained values ~0.3 nmol N L^{-1} h^{-1}

westwards until ~32°W and then decreased steadily towards the western end of the transect (Table I).

Longitudinal distribution of diazotrophic phylotypes

The different diazotrophic phylotypes showed variability in their longitudinal distribution, with a shift in their patterns occurring at ~30°W (Fig. 2). *Trichodesmium* (only assayed in the >10 μm fraction) had the highest *nifH* copy L^{-1} numbers detected overall in this study, showing low abundances east of ~30°W, but increasing from ~10^4 *nifH* copies L^{-1} to a maximum of 8.8×10^5 *nifH* copies L^{-1} towards the western end of the transect (Fig. 2a). *Trichodesmium* trichomes (as counted by the FlowCam from samples recovered with a plankton net, see Methods) were absent between station 1 and ~15°W, peaked at ~28°W with ~3×10^5 trichomes L^{-1} and then decreased steadily until $7-9 \times 10^3$ trichomes L^{-1} at the westernmost stations of the transect (Fig. 2a).

The putative Gammaproteobacterium γ-24774A11 was detected in the >10 and 10−3 μm fractions, with maximum abundances in the former up to 3.3×10^3 *nifH* copies L^{-1}, although their longitudinal distribution and abundance were very similar in both size fractions (Fig. 2a and b). In the >10 μm fraction, γ-24774A11 showed a stable longitudinal pattern at 10^2 *nifH* copies L^{-1}, whereas in the 10−3 μm fraction, their distribution was similar to that of *Trichodesmium*, with non-detectable abundances at longitudes east of ~30°W and an increasing pattern west of this point, stabilizing at 10^2 *nifH* copies L^{-1} until 80°W (Fig. 2b). γ-24774A11 were not detected in the 3−0.2 μm fraction (Fig. 2c). Het1 were detected at low abundances ($6.2 \times 10^1-3.4 \times 10^2$ *nifH* copies L^{-1}; Fig. 2a and b) at stations located east of ~30°W, with the exception of station 104 at 62°W, where they were detected at 3.8×10^1 *nifH* copies L^{-1} (Fig. 2a). The abundance of UCYN-A decreased longitudinally from east to west (Fig. 2b and c). They were detected from the easternmost station until ~50°W in the 10−3 μm fraction (Fig. 2b), but only until ~30°W and at higher abundances (~10^4 *nifH* copies L^{-1}) in the 3−0.2 μm fraction (Fig. 2c). UCYN-B were only detected in one sample (station 91, 54°W) at a low abundance (~10^2 *nifH* copies L^{-1}).

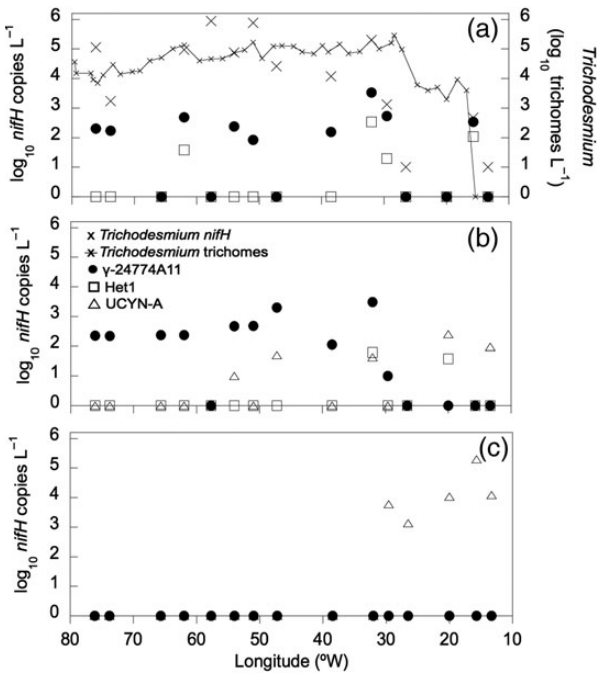

Fig. 2. Abundance of the diazotrophic phylotypes (*nifH* copies L^{-1}) *Trichodesmium*, γ-24774A11, Het1 and UCYN-A retained on (**a**) the >10 μm, (**b**) 3 μm and (**c**) 0.2 μm fractions. The abundance of *Trichodesmium* trichomes ($\log_{10}$ trichomes L^{-1}) is superimposed on (a) and scale shown on the right *y*-axis. 163 × 183 mm^2 (200 × 200 DPI).

Correlations with environmental variables and east-west differences

The significant Spearman correlations ($P < 0.00042$ after applying a Bonferroni correction for multiple comparisons; McDonald, 2014) between the abundance of diazotrophic phylotypes with environmental parameters (temperature, salinity, oxygen, fluorescence and nutrients) are shown in Table II. Some significant correlations were found between different diazotrophs. UCYN-A from the 3−0.2 μm fraction were negatively correlated with γ-24774A11 from the 10−3 μm fraction and with *Trichodesmium nifH* copies (analysed only in the >10 μm fraction). Interestingly, *Trichodesmium nifH* copies were not significantly correlated with their corresponding trichome counts, although visually some correspondence was apparent in the two methods (Fig. 2a). The latter were instead correlated positively with γ-24774A11 (in both the >10 and 10−3 μm fractions).

Some diazotrophs also showed significant correlations with environmental parameters. A negative correlation was observed between temperature and the *nifH* gene copies of UCYN-A from the 3−0.2 μm fraction. Positive correlations were also observed between salinity and *Trichodesmium* trichome counts, and hence the correlation of the latter was negative with temperature (temperature and salinity were negatively correlated). UCYN-A recovered from the 3−0.2 μm fraction were positively correlated with oxygen and fluorescence (note that fluorescence and oxygen were correlated). No significant correlations were found with inorganic nutrients.

If we divide the transect into two halves (west and east of 27.15°W or station 37) and pool the observations

Table II: Significant ($P < 0.00042$) Spearman correlations (positive or negative) between diazotroph abundances, environmental variables and nutrient concentrations

	UCYN-A 3 µm	UCYN-A 0.2 µm	γ-24774A11 10 µm	γ-24774A11 3 µm	*Trichodesmium nifH*	*Trichodesmium* counts	Het1 10 µm	Het1 3 µm	Temperature	Salinity	Oxygen	Fluorescence	Nitrate + nitrite	Phosphate	Silicate
UCYN-A 3 µm															
UCYN-A 0.2 µm				−	−				−		+	+			
γ-24774A11 10 µm						+	+								
γ-24774A11 3 µm						+									
Trichodesmium nifH															
Trichodesmium counts										+					
Het1 10 µm															
Het1 3 µm															
Temperature										−	−	−			
Salinity												+			
Oxygen															
Fluorescence															
Nitrate+nitrite															
Phosphate															+
Silicate															

within each of the halves, steep gradients in *nifH* abundances are demonstrated: the abundance of UCYN-A in the 3−0.2 µm fraction, γ-24774A11 in the 10−3 µm fraction and *Trichodesmium nifH* and filament counts were significantly different between the eastern and western halves (Wilcoxon test $P = 0.05$, 0.02, 0.006 and 0.009, respectively).

DISCUSSION

Longitudinal variability of diazotroph phylotypes

Our results suggest that the subtropical Atlantic Ocean has a clear east-west divide at ∼30°W, where the productivity of surface waters decreases as depicted by Chl *a* concentrations in Fig. 1, the deep chlorophyll maximum deepens and the presence of measurable phosphate concentrations within the euphotic zone disappears (Table I; see also Fig. 2 in Mompeán *et al.*, 2013). This is a physical separation caused by the transition from the upwelling-influenced waters off the Northwest African coast to the NASG, which affects the structure and activity of planktonic communities (e.g. Hernández-León *et al.*, 1999). Our data show that this physical barrier also affects diazotrophs, in a phylotype-specific manner.

Trichodesmium was the most abundant diazotroph phylotype detected by qPCR assays along the transect, followed by UCYN-A and γ-24774A11, in agreement with previous studies in the North Atlantic (e.g. Langlois *et al.*, 2008; Ratten *et al.*, 2015). *Trichodesmium* was found at low abundances east of ∼30°W estimated by either qPCR or filament counts (Fig. 2a), agreeing with previous studies (Hernández-León *et al.*, 1999; Fernández *et al.*, 2012), and increased towards the west in parallel with increasing temperature and a shoaling MLD (Table I), consistent with its requirements for high light and oligotrophic conditions (Carpenter and Roenneberg, 1995). *Trichodesmium* is known for most frequently thriving in warm, stratified and nitrate-poor waters (Capone *et al.*, 2005), whereas in the colder and nutrient-richer waters of the eastern subtropical and tropical North Atlantic, it is almost absent or present at very low abundances and mainly found as free trichomes instead of colonies (Fernández *et al.*, 2010; Goebel *et al.*, 2010; Benavides *et al.*, 2011).

In this study, we determined the abundance of *Trichodesmium* in two ways: by quantification of *nifH* gene copies in seawater samples collected from the 5 m depth with Niskin bottles and filtered onto 10 µm filters (>10 µm fraction) and by vertically sampling the water column through the top 200 m using a 40 µm mesh net, followed by counting trichomes with a FlowCam (see Methods). Although the overall east-west trend was

similar, *Trichodesmium nifH* copy numbers did not correlate significantly with *Trichodesmium* trichome counts (Table II and Fig. 2a). When dividing *Trichodesmium nifH* copy numbers by trichome counts at stations where both types of data were available, each trichome would have contained between 0.7 and 4.5 *nifH* copies (with an exception of 19 *nifH* copies per filament at station 97; data not shown). This is one to two orders of magnitude fewer than would be expected, given that a 100 μm filament has approximately 70 cells (Tyrrell *et al.*, 2003). The number of cells per trichome is variable (usually up to 100 cells, but finding broken filaments with fewer cells is common; Benavides *et al.*, 2011). Parallel comparison of the two counting methods was only possible for six stations, and the differences between these methods may have affected the results considerably. Plankton nets are generally preferred over Niskin bottles for sampling *Trichodesmium* when its abundance is low (Chang, 2000), given the enormous difference in the volume of seawater filtered in each case (tens of litres from Niskin bottles versus hundreds to thousands of litres concentrated when using net tows). The differences observed between the two counting methods are also likely associated with the sampling depths covered (5 m versus integrated over 200 m), as well as the vertical migration of *Trichodesmium* colonies (Villareal and Carpenter, 2003), and/or their patchy distribution in the water column (Carpenter *et al.*, 2004; Davis and McGillicuddy, 2006).

UCYN-A detected in the 10−3 μm fraction were more abundant east of ∼30°W and only detected in the 3−0.2 μm fraction from samples collected between ∼30°W and the Northwest African coast (Fig. 2b and c). UCYN-A were the second most abundant phylotype in our study, highlighting their potential role in bulk N_2 fixation activity. UCYN-A usually appear in symbiosis with a prymnesiophyte from which it obtains organic carbon (Thompson *et al.*, 2012), although it has also been found in association with larger plankton cells such as diatoms and dinoflagellates (Thompson *et al.*, 2014), and at times as free-living cells, likely due to their loose association with the surface of their host (Thompson *et al.*, 2012; Krupke *et al.*, 2014). These different association possibilities, as well as detachment during sample handling, would explain the presence of UCYN-A in both the 10−3 and the 3−0.2 μm fractions in our study (Fig. 2b and c), which is consistent with previous reports (Agawin *et al.*, 2014). Unidentified diazotrophic cells in the UCYN-A size range were also observed in aggregates using whole-cell hybridization techniques (Benavides *et al.*, 2013a), which could contribute to their gene copies being found in the 10−3 μm size fraction in this study. The association of UCYN-A with phytoplankton cells also likely explains their apparent preference for colder and nutrient-rich waters compared with other diazotrophs (Moisander *et al.*, 2010). In the North Atlantic, UCYN-A have been found in waters with temperatures ranging from ∼12°C or lower to 23°C (Langlois *et al.*, 2008; Rees *et al.*, 2009; Bentzon-Tilia *et al.*, 2014; Krupke *et al.*, 2014; Scavotto *et al.*, 2015). UCYN-A have also been reported as the dominant diazotroph in the upwelling waters off the Northwest coast of Africa with temperatures as low as 17°C and high nutrient concentrations (Agawin *et al.*, 2014), as well as over the Equatorial upwelling waters where similar conditions are found (Foster *et al.*, 2009). Although active UCYN-A (as *nifH* transcripts) were not found in these cold and nutrient-rich waters (e.g. Foster *et al.*, 2009), the higher N_2 fixation activity off the Northwest coast of Africa (Benavides *et al.*, 2013b) and in the Equatorial upwelling where they dominate (Subramaniam *et al.*, 2013) suggests that they contribute importantly to fixed nitrogen inputs in these areas. In our study, UCYN-A were detected at $>10^4$ *nifH* gene copies L^{-1} in waters with temperatures ranging between ∼19 and 21°C, with very high nutrient concentrations (e.g. nitrate plus nitrite >12 μM; Table I). However, although these diazotrophs have been detected in higher latitude coastal waters (off the Northeast American coast; Mulholland *et al.*, 2012), their ubiquitous presence in oceanic waters at latitudes >30−40°N was suggested to be unlikely (Krupke *et al.*, 2014). The clear longitudinal segregation in UCYN-A abundances may also be driven by iron-stress alleviation via Saharan dust inputs, which are higher in this longitudinal range of the transect (Benavides *et al.*, 2013b) and were reported to limit N_2 fixation by UCYN-A (Krupke *et al.*, 2015).

Non-cyanobacterial diazotrophs are cosmopolitan, and their activity may be driven by factors other than those controlling cyanobacterial diazotrophs (Farnelid *et al.*, 2011). Interestingly, during our study, γ-24774A11 *nifH* copies were detected at abundances comparable to those of UCYN-A (Fig. 2). The γ-24774A11 have been shown to consistently express their *nifH* gene in the open ocean (Moisander *et al.*, 2014), suggesting active N_2 fixation. Thus, if their per cell N_2 fixation activities are as high as with UCYN-A, with equal cell numbers they might have a comparable contribution to *in situ* N_2 fixation rates. The γ-24774A11 were found exclusively in the >10 and 10−3 μm size fractions, implying the attachment of these diazotrophs to larger microbes, particulate material or a self-production of organic aggregates. The association of non-cyanobacterial diazotrophs with particles as a means of obtaining organic nutrients has been previously hypothesized, and gammaproteobacterial representatives have been found forming aggregates in oxygenated cultures (Bentzon-Tilia *et al.*, 2015).

A particle-attached mode of life may provide several advantages for planktonic bacterial diazotrophs, such as oxygen-depleted microzones and the availability of organic matter (Riemann *et al.*, 2010). However, the longitudinal distribution of Chl *a* along the transect (Fig. 1) suggests that organic particles were more abundant east of ~30°W than on the western half of the transect, which is opposite to the distribution of γ-24774A11 in the >10 and 10–3 μm fractions (Fig. 2a and b). This makes the self-production of organic aggregates the most plausible explanation for their longitudinal distribution in our study, which is consistent with a previous study (Bentzon-Tilia *et al.*, 2015). Nevertheless, the association of diazotrophic Gammaproteobacteria with pelagic particles has not been studied directly, and we cannot assume that the colonization of particles by these microbes is equal in space and time; it is possible that it changes with particle size, chemical composition, among other characteristics. The filtration method we used may have contributed to the observation of high abundances of γ-24774A11 in the >10 and 10–3 μm size fractions. In our past studies in which a peristaltic pump system was used, this phylotype was not detected in the >10 μm size fraction (Moisander *et al.*, unpublished data). It is possible that the water jet vacuum aspirator filtration method used here is more gentle than the peristaltic pump system and generates less bacterial detachment from particles or aggregates. The high abundance of γ-24774A11 detected together with previous studies (Moisander *et al.*, 2014; Langlois *et al.*, 2015) underscores the current need to quantify the contribution of non-cyanobacterial diazotrophs to global N_2 fixation rates, as well as to elucidate their ecology and physiological constraints. At present, it is our inability to discern cyanobacterial from non-cyanobacterial N_2 fixation activity that keeps us from further estimating the global importance of the latter (Benavides and Voss, 2015), although the measurable diazotrophic activity in aphotic waters (e.g. Bonnet *et al.*, 2013), as well as the enhancement of N_2 fixation rates upon the addition of photosystem II-blocking agents to stop the activity of autotrophic diazotrophs (Rahav *et al.*, 2015), suggests that their activity may be significant.

Similar to UCYN-A, Het1 diazotrophs (*Richelia*) depend on the ecological constraints of their host (*Rhizosolenia* diatoms). Over the transect surveyed in this study, Het1 were found at low abundances east of ~30°W in the NASG boundary area (Fig. 2), coinciding with the highest phosphate and silicate concentrations of the transect because of the proximity of the Northwest African coastal upwelling (Table I). Het1 are usually restricted to a river plume or coastal runoff-affected areas such as the Amazon and the Congo River plumes (Foster *et al.*, 2007, 2009) and the North American coast at higher latitudes (e.g. >30°N; Ratten *et al.*, 2015). Their maximum abundances measured during our study (~10^2 *nifH* copies L^{-1}; Fig. 2) are below those in the Amazon and Congo River plumes (up to 10^5 and 10^3 *nifH* copies L^{-1}, respectively; Foster *et al.*, 2007, 2009), and other studies performed in open-ocean waters of the North Atlantic have generally also found low abundances (<10^1 *nifH* copies L^{-1}; Luo *et al.*, 2012; also see Fig. 3 in Benavides and Voss, 2015). Overall, the low abundances and the low frequency of detection among samples over the transect suggest that their contribution to bulk N_2 fixation in this area is minimal. It also needs to be noted that although the *nifH* gene copies are detected in each cell of heterocystous diazotrophs, only some of the cells are heterocysts and thus N_2-fixing. If only the number of N_2-fixing cells is considered, the numbers reported here should be divided by the estimated vegetative cell: heterocyst frequency, which for *Richelia* has been reported to be approximately 5 (Foster *et al.*, 2009). Taking into account, this conversion would reduce the contribution of this group to N_2 fixation further.

Relationship between the diazotrophic community and N_2 fixation rates along 24.5°N

The longitudinal variability of N_2 fixation activity in the North Atlantic (encompassing both its eastern and western basins) has only been addressed in a few studies (Voss *et al.*, 2004; Montoya *et al.*, 2007; Benavides *et al.*, 2013b; Ratten *et al.*, 2015). The size-fractionated (>10 and <10 μm) N_2 fixation rates corresponding to water samples investigated in this study have been published previously (Benavides *et al.*, 2013b) and are also shown in Table I. N_2 fixation rates in both fractions decreased from east to west from ~0.4 to 0.01 nmol N L^{-1} h^{-1}. This pattern is opposite to what would be expected from a *Trichodesmium*-dominated community; indeed, higher rates towards the western Atlantic have been observed previously in studies focusing on these cyanobacteria (e.g. Capone *et al.*, 2005), but other bulk water N_2 fixation studies found an opposite longitudinal pattern (e.g. Voss *et al.*, 2004). Benavides *et al.* (2013b) divided the transect into eastern and western halves at about ~45°W, coinciding with a change in sea surface height values. Despite this division that resulted in significantly different N_2 fixation rates between the eastern and western halves of the transect, the visual inspection of N_2 fixation rates (Table I) shows that the steepest change in N_2 fixation activity occurs at ~32°W, agreeing with the shift observed in the composition of the diazotrophic community in this study (Fig. 2).

Prior reports suggest that in tropical latitudes, UCYN-A dominate the N_2 fixation activity in the eastern North Atlantic basin, whereas *Trichodesmium* dominate in the western basin (Montoya *et al.*, 2007; Benavides and Voss, 2015). This study suggests that a similar trend in *nifH* phylotype distributions of UCYN-A and *Trichodesmium* is found also in subtropical latitudes. The distribution of *nifH* copies in the different size fractions measured suggests that in subtropical latitudes Gammaproteobacteria may also contribute importantly to >3 μm N_2 fixation in the western North Atlantic basin. The presence of small diazotrophs such as Gammaproteobacteria in the >10 and 10−3 μm size fractions suggests that additional size fractionation of samples incubated with $^{15}N_2$ with 3 μm filters could provide further information about groups contributing to the N_2 fixation rates.

Over our transect, <10 μm N_2 fixation rates contributed on average 40% to bulk N_2 fixation (Benavides *et al.*, 2013b), suggesting an important role of the diazotrophs captured on the 10−3 and 3−0.2 μm fractions and quantified in this study. Moreover, <10 μm N_2 fixation rates were significantly higher in the eastern half of the transect when compared with the western half, supported by the high abundance of UCYN-A detected (Fig. 2). In summary, N_2 fixation rates may be driven primarily by UCYN-A in the eastern part of the transect and by a combination of *Trichodesmium* and γ-24774A11 in the western part.

CONCLUSIONS

This study covered two areas representative of the conditions at the boundary and centre of the NASG and provides a description of the diazotrophic community in an undersampled area of the North Atlantic Ocean. Our results indicate that the transition between the boundary and central NASG creates a separation in the composition of the diazotrophic community, with UCYN-A dominating the eastern half of the Atlantic and *Trichodesmium* and γ-24774A11 Gammaproteobacteria dominating the western part.

The detection of γ-24774A11 in the >10 and 10−3 μm fractions and not in the 3−0.2 μm fraction indicates that these diazotrophs occur in association with either larger microbes, existing marine particles, or self-produced mucilage matrices; to our knowledge, this has not been previously reported. The filtration method we used may be more gentle than a peristaltic pump system, having less bacterial detachment from particles. These results also suggest that size-fractionation studies are not optimal for identifying the different diazotroph phylotypes responsible for the measured N_2 fixation rates and support past observations that non-cyanobacterial diazotrophs are present at considerable abundances in open-ocean waters, thus deserving further study.

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